

DESCRIPTION**Drug Screening Method**

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Technical Field

10 The invention of this application relates to a novel method of screening a drug for obesity and/or diabetes by using the expression and the activity of a ligand factor ERRL1 for a nuclear receptor ERR (estrogen receptor- related receptor) as an index.

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Background Art

The most typical molecular mechanism that underlies gene expression regulated by nuclear receptors starts from the binding of their ligands (e.g., small lipophilic molecules such as steroids, retinoic acid, thyroid hormone and vitamin D₃) (Mangelsdorf, D. J. et al., Cell 83: 835-839, 1995). Endogenous levels of such ligands are strictly regulated by means of multiple enzymatic reaction steps working toward their production and/or degradation (Honkakoski, P. & Negishi, M. Biochem. J. 347: 321-337, 2000). In addition, these ligands are collectively called lipophilic ligands or lipophilic hormones of the endocrine system. By changing ligand levels, the endocrine system contributes to the adaptation to changes in the in vitro or in vivo environment, namely to homeostasis (Mangelsdorf, D. J. et al., Cell 83: 835-839, 1995; Giguere, V. Endocr. Rev. 20: 689-725, 1999). This system appears advantageous for slow and long-term adaptations but

disadvantageous for quick responses because of the complicated regulation of ligand production. On the other hand, genome analysis has predicted the existence of numerous nuclear receptor-like molecules. However, the lipophilic ligands specific to the molecules are currently hardly known, and these molecules are collectively called orphan receptors (Giguere, V. *Endocr. Rev.* 20: 689-725, 1999). The activation mechanisms of orphan receptors remain totally unknown. Estrogen receptor-related proteins 1 and 2 (ERR1 and -2) were the first identified orphan receptors (Giguere, V. et al., *Nature* 331: 91-94, 1988; Shigeta, H. et al., *J. Mol. Endocrinol.* 19: 299-309, 1997), and its third member (ERR3) has recently been isolated (Eudy, J. D. et al., *Genomics* 50: 382-384, 1998; Hong, H. et al., *J. Biol. Chem.* 274: 22618-22626, 1999). ERRs and estrogen receptors share structural similarity, however, ERRs do not respond to estrogen (Giguere, V. *Endocr. Rev.* 20: 689-725, 1999). On the other hand, ERR1 has been proposed to act as a key transcriptional regulator of the gene encoding medium-chain acyl CoA dehydrogenase (MCAD), namely a pivotal enzyme in mitochondrial fatty acid β -oxidation (Sladek, R. et al., *Mol. Cell. Biol.* 17: 5400-5409, 1997; Vega, R. B. & Kelly, D. P., *J. Biol. Chem.* 272: 31693-31699, 1997). These observations lead to the idea that ERR-mediated gene regulation may play important roles in the control of energy balance in the body by regulating fatty acid β -oxidation, which is generally induced by the physical exercises (Horowitz, J. F. & Klein, S., *Am. J. Clin. Nutri.* 72: 558S-563S, 2000). Therefore, daily performance of appropriate physical exercises is considered to be the simplest and most effective way to cope with obesity and diabetes (Baldwin, K. M., *J. Appl. Physiol.* 88: 332-336, 2000).

As described above, a nuclear orphan receptor, ERR serves as a regulator for MCAD gene expression, and it is considered that the

resistance to obesity or diabetes can be maintained by controlling energy balance in the body by regulating fatty acid β -oxidation by this MCAD gene expression. However, the ligand molecule for the receptor ERR which is the origin of a series of such mechanisms has not been identified yet.

On the other hand, by researchers in the nuclear receptor field, it has been revealed that several classes of transcriptional cofactor proteins, such as SRC1/p160 family (Onate, S. A. et al., Science 270: 1354-1357, 1995), P/CAF (Blanco, J. C. G. et al., Genes Dev. 12: 1638-1651, 1998), and CBP/p300 (Chakravarti, D. et al., Nature, 383: 99-103, 1996; Kamei, Y. et al., Cell 85: 403-414, 1996) play key roles in ligand-dependent transcriptional activation of the nuclear receptors. These are called coactivators and are ubiquitously expressed. Further, their expression levels appear not to change during differentiation of cells or in response to changes in external and internal environments. More recently, a unique coactivator, termed PPAR γ -coactivator-1 (PGC-1) was identified (Puigserver, P. et al., Cell, 92: 829-839, 1998). This coactivator distinguishes itself from other coactivators by means of its tissue-specific and regulated expression. In other words, PGC-1 is expressed in different levels in brown adipose tissue (BAT), skeletal muscle, heart, kidney, and brain and the expression is markedly up-regulated in BAT after acute exposure to cold stress (Puigserver, P. et al., Cell, 92: 829-839, 1998). PGC-1 expression is also up-regulated in the liver (Yoon, J. C. et al., Nature, 413: 131-138, 2001) and heart (Lehman, J. J. et al., J. Clin. Invest. 106: 847-856, 2000) under fasting conditions.

PPAR γ is known to be the key regulator of adipogenesis (Tontonoz, P. et al., Cell 79, 1147-1156, 1994), and its expression is augmented during the differentiation of adipocytes. In addition, it has been

revealed that thiazolidine (TZD), which has been developed as an improving agent for insulin resistance in diabetes, serves as the ligand for PPAR γ (Lehmann, et al., J. Biol. Chem. 270: 12953-12956, 1995), and screening of novel drugs for diabetes using the transcriptional activation of PPAR γ as an index has been widely carried out.

However, the level of PGC-1 mRNA, which is a coactivator of PPAR γ , is at a very low level during adipocyte differentiation of 3T3-L1 cells. Therefore, it was speculated that a similar molecule to PGC-1, which may function during adipocyte differentiation might exist. The inventors of this application identified a novel binding factor, PGC2, to PPAR γ , and has filed a patent application (JP-A-2002-058489) for the method of screening a ligand which regulates the transcriptional activity of PGC2-PPAR γ complex (in particular, a ligand for decreasing activity of PGC2-PPAR γ complex) as a means for developing a drug for insulin resistance diabetes.

Disclosure of the invention

The inventors of this application searched for a ligand factor for a nuclear receptor ERR, a regulator for the expression of MCAD which is a resistance gene to obesity or diabetes. As a result, they found out that this factor is PGC2 which had been identified before as a ligand factor for PPAR γ , and named this protein factor ERRL1 (abbreviation of ERR ligand 1).

The invention of this application is based on such a novel finding by the inventors, and makes it an object to provide a method of screening a drug targeting an endogenous pathway of anti-obesity and

anti-diabetes related to the nuclear receptor ERR and its target gene MCAD, which is totally different from the action mechanism of PPAR γ .

In order to solve the foregoing object, this invention provides a method of screening a substance which serves as the active ingredient in a drug for obesity and/or diabetes, which comprises treating cells or an animal with a candidate substance and specifying a candidate substance fulfilling one or more of the following requirements as the target active ingredient substance:

- (a) increasing the expression level of a ligand factor ERRL1 for a nuclear receptor ERR;
- (b) increasing the transcriptional activity of the nuclear receptor ERR;
- (c) promoting the binding of ERRL1 to ERR; and
- (d) increasing the expression level of an MCAD gene product.

In addition, this invention provides a drug for obesity and/or diabetes comprising as the active ingredient one or more substances specified by the foregoing screening method.

Further, this invention provides a transgenic non-human animal having a purified polynucleotide encoding a ligand factor ERRL1 for a nuclear receptor ERR in its genomic DNA and overexpressing the ligand factor ERRL1.

In this invention, 'polynucleotide' means a molecule in which phosphate esters of nucleosides in which a purine or a pyrimidine has been bound to a sugar via a β -N-glycosidic bond (ATP, GTP, CTP, UTP; or dATP, dGTP, dCTP or dTTP) have been bound to one another. Specific examples include a genomic DNA encoding a protein, an mRNA

transcribed from the genomic DNA and a cDNA synthesized from the mRNA. It may be either a double strand or a single strand. Further, a sense strand and an antisense strand of these genomic DNA, mRNA and cDNA are included. In addition, 'protein' and 'peptide' mean a molecule
5 composed of a plural number of amino acid residues bound one another via an amide bond (peptide bond).

The terms and concepts in this invention will be defined in detail in the description of the embodiments or Examples of the invention. In
10 addition, various techniques used for implementing this invention can be easily and surely carried out by those skilled in the art based on known literatures or the like except for the techniques whose sources are particularly specified. For example, preparation of a drug is described in Remington's Pharmaceutical Sciences, 18th Edition, ed. A. Gennaro,
15 Mack Publishing Co., Easton, PA, 1990, and techniques of genetic engineering and molecular biology are described in Sambrook, and Maniatis, in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989; Ausubel, F. M. et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y, 1995
20 and the like.

Brief Description of Drawings

25 Fig. 1 shows the comparison of the primary amino acid sequences between ERRL1 and PGC-1. Asterisks indicate identical amino acids. The numbers represent the amino acid positions with the first methionine counted as one. LXXLL motifs are shown in bold types. Glutamic acid (E) repeats and serine-arginine (SR)-rich regions are
30 underlined. A putative RNA-binding motif is boxed. Tentative domain

borders are shown by vertical lines. A splicing variant of ERRL1, which was newly identified at this time, lacks 39 amino acids (from the 156th leucine to the 194th lysine).

5 Fig. 2 shows the expression profiles of ERRL1 mRNA. Fig. 2A shows the results of analyzing 20 µg of total RNA from various adult mouse tissues using ERRL1, PGC-1, PRC, ERR1 and ERR3 cDNAs as probes. Ethidium bromide staining of 28S RNAs is also shown. Fig. 2B shows the results obtained by inducing 3T3-L1 cells to differentiate into
10 adipocytes by the treatment with dexamethasone, 1-methyl-3-isobutylxanthin and insulin (added day 0), isolating RNAs and performing the Northern blot analysis. The blot was sequentially hybridized with the respective probes. Fig. 2C shows the results of observing the expression of ERRL1, ERR1 and MCAD mRNA in 3T3-L1
15 and 10T1/2 cells (-: preadipocytes, +: mature adipocytes).

 Fig. 3 shows the profiles of PGC-1 and ERRL1 as 'protein ligands' for various nuclear receptors. Transcriptional activation ability of PGC-1 (A) and ERRL1 (B) for various nuclear receptors was examined in
20 a transfection assay. Mean values of triplicate experiments are shown as fold induction, where the Luc activity of each GAL4-fused nuclear receptor in the absence of PGC-1 or ERRL1 serves as the reference values. Error bars show standard deviation. Fig. 3C shows the dose-dependent activity of ERRL1 on the full-length ERR-mediated
25 transcription via ERRE. pCMX-ERRL1 in different amounts (0 to 200 ng) was co-transfected with 30 ng of pCMX-ERR1 or pCMX-ERR3 in the presence of 250 ng of TK-Luc or ERRE-Luc in CV-1 cells. Mean values from triplicate experiments are shown as fold induction, where the Luc activity of TK-Luc in the absence of ERRL1 serves as the reference value.
30 Error bars show standard deviation. Fig. 3D shows the results of

confirming that ³⁵S-labeled ERRL1 shows strong interaction with GST-ERR1 and GST-ERR3 in vitro. Autoradiographs show the pulled down proteins (GST-ERR1 and GST-ERR3) and 10% of the total amount of ³⁵S-labeled ERRL1 used for the GST pull-down assay (Input). CBB staining of the SDS-PAGE gel containing GST-fused proteins used in this assay is also presented. Fig. 3E shows the results showing augmentation of MCAD mRNA expression by ectopic expression of ERRL1 or PGC-1 in combination with ERR1 or ERR3 in 10T1/2 cells. One day after confluence of the cells, total RNA was isolated, and analyzed by Northern blot (20 µg per lane) using MCAD and ERRL1 cDNAs as probes. The relative densitometric values of MCAD mRNAs are also included beneath each lane.

Fig. 4 relates to the creation of ERRL1 transgenic mouse. Fig. 4A shows the schematic drawing of the ERRL1 transgene and the positions of probes used for Southern (probe 1) and Northern blots (probe 1 and probe 2). Fig. 4B shows the results of showing that in the two transgenic lines (A1 and A2), 10 and 12 copies of each of the transgenes were contained, respectively. Fig. 4C shows the results of Northern blot analysis of ERRL1 mRNA expression in the respective tissues from ERRL1 mice (line A1) and littermate control mice. Each lane contained 20 µg of total RNA. Fig. 4D shows the results of measuring the expression of ERRL1, MCAD, ACC2 and UCP-3 in the skeletal muscle of ERRL1 transgenic mice and wild-type control mice. Three mice in each group were examined. Quantitative values of Northern signals (control as 100%) are shown on the right. Data are mean ± s.e.m. (Asterisk shows $p < 0.05$).

Fig. 5 relates to the phenotypes of ERRL1 transgenic mice. Fig. 5A shows the cumulative food intake per mouse during the indicated

period by measuring food intake every week. Data are means \pm s.e.m. (n = 6 per animal group, error bars are smaller than the symbols, asterisk shows $P < 0.05$, double asterisk shows $P < 0.01$). Fig. 5B shows the results of showing the changes in the body weight of ERRL1 transgenic mice (closed circle) and wild-type controls (open circle). Values represent mean body weight \pm s.e.m. (n = 6 per animal group, asterisk shows $P < 0.05$, double asterisk shows $P < 0.01$). For some data points, the error bars are smaller than the symbols. Fig. 5C shows the results of reduction of abdominal fat in ERRL1 transgenic mice. Fig. 5D shows the comparison of epididymal WAT weight between wild-type control mice and ERRL1 transgenic mice. The columns represent mean values of WAT weight \pm s.e.m. (n = 6 per animal group, asterisk shows $P < 0.05$). Fig. 5E shows the comparison of the morphology of white adipose tissue between ERRL1 transgenic mice and littermate wild-type control mice. The scale bar indicates 50 μ m. Fig. 5F shows the average diameter of adipose cells. The diameters of the cells were measured from the sections shown in Fig. 5E (n = 20, double asterisk shows $P < 0.01$). Figs 5G and 5H show the results of showing energy expenditures at resting (G) and total (H) in 12-week-old control mice and ERRL1 transgenic mice. The columns represent mean values of energy expenditure \pm s.e.m. (n = 6 per animal group, asterisk shows $P < 0.05$, double asterisk shows $P < 0.01$).

Fig. 6A shows the results of measuring changes in body weight of KKAY(+)ERRL1(-) (open square, n = 8), KKAY(-) ERRL1(-) (open circle, n = 5), KKAY(+)ERRL1(+) (closed square, n = 5), KKAY(-)ERRL1(+) (closed circle, n = 5) male mice. The body weight curve of each group was compared by repeated-measure analysis (Super ANOVA). Asterisk shows $P < 0.05$, double asterisk shows $P < 0.01$. Fig. 6B shows representative photographs of 12-week-old male KKAY(+)ERRL1(-) (left),

KKAy(+)ERRL1(+) (center) and KKAy(-)ERRL1(-) (right) mice.

Best Mode for Carrying Out the Invention

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The ligand factor ERRL1 in the current invention is, for example, mouse ERRL1 (SEQ ID NO: 2) which has been disclosed as 'mouse PGC2' in SEQ ID NO: 1 in JP-A-2002-058489. Further, as for this ligand factor ERRL1, its splicing variant (the one lacking 39 amino acids from the 156th leucine to the 194th lysine of SEQ ID NO: 2) can be used as a target. In addition, known human PERC (PPAR γ coactivator β 1: Entrez protein database No. NP_573570), which is a human homologue to mouse PGC2, can be also used as a target of this invention for a human ERRL1. As the polynucleotide encoding mouse ERRL1, mouse PGC2 cDNA (ERRL1 cDNA: SEQ ID NO: 1) whose nucleotide sequence has been disclosed in SEQ ID NO: 2 in JP-A-2002-058489 can be used. Further, as the polynucleotide encoding human ERRL1, the foregoing human PERC cDNA (GenBank No. NM_133263) can be used. Still further, for example, human PRRC gene exists in chromosome 5q33.1 region, and its genomic sequence is publicly known (GenBank No. NT_029289). Therefore, this invention can be carried out by utilizing a promoter region existing in this genomic gene sequence.

With regard to the nuclear receptor ERR of this invention, any one or more selected from ERR1, ERR2 and ERR3 (Giguere, V. et al., Nature 331: 91-94, 1988; Shigeta, H. et al., J. Mol. Endocrinol. 19: 299-309, 1997; Eudy, J. D. et al., Genomics 50: 382-384, 1998; Hong, H. et al., J. Biol. Chem. 274: 22618-22626, 1999) can be used as a target. In addition, cDNAs encoding them, respectively, are also publicly known. For example, ERR1 cDNA is known on GenBank No. NM_004451, and the

genomic gene (chromosome 11q13 region) sequence is known on GenBank No. NT_033903. Therefore, this invention can be carried out by using these polynucleotides (cDNA, genomic DNA, a DNA fragment of the gene promoter region and the like). Further, as for MCAD gene, its cDNA is also publicly known (GenBank No. AF251043).

The foregoing polynucleotides can be isolated by screening a genomic DNA library or a cDNA library using, as a probe, a DNA fragment consisting of a known nucleotide sequence or its partial sequence, respectively. The obtained polynucleotide can be amplified by a gene amplification method, which is usually performed, such as the PCR (polymerase chain reaction) method, NASBN (nucleic acid sequence based amplification) method, TMA (transcription-mediated amplification) method or SDA (strand displacement amplification) method. In addition, in the case of cDNA, by using a primer synthesized based on a known sequence, a target cDNA can be obtained by also the RT-PCR method in which mRNA isolated from the cell is used as a template.

The method of screening a substance which serves as the active ingredient in a drug for obesity and/or diabetes of this invention comprises treating cells or an animal with a candidate substance and specifying a candidate substance fulfilling one or more of the following requirements as the target substance by using the foregoing protein factors or polynucleotides encoding them:

- (a) increasing the expression level of a ligand factor ERRL1;
- (b) increasing the transcriptional activity of a nuclear receptor ERR;
- (c) promoting the binding of ERRL1 to ERR; and
- (d) increasing the expression level of an MCAD gene product.

In other word, as explained above, a nuclear receptor ERR serves

as a regulator for MCAD gene expression, and energy balance in the body is controlled by regulating fatty acid β -oxidation by this MCAD gene expression, whereby the resistance to obesity or diabetes can be maintained. As shown in the Examples described later, ERRL1 is a
5 ligand molecule for the receptor ERR which is the origin of a series of such mechanisms. Indeed, it was confirmed that augmentation of ERRL1 expression shows a significant effect on acquisition of the resistance to obesity or diabetes in mouse. In other words, a substance fulfilling the foregoing requirements (a) to (d) is a substance which acts
10 on the process of anti-obesity and anti-diabetic mechanism.

In the methods of this invention, a 'candidate substance' includes an organic or inorganic substance, a protein, a peptide, a polynucleotide, an oligonucleotide and the like, which are either unknown or known.
15 'Cells' are ones isolated from an established human cell line or non-human animal cell line or from a tissue of a human or a non-human animal body and appropriately maintained in a culture condition. Examples of the established cell line include an adipocyte such as 3T3-L1 cell line. As described in Examples below, because the
20 expression of ERRL1 protein shows a sensitive response during the differentiation of this adipocyte, those are preferred cells for the search for a target substance. Alternatively, the cells may be an established cell line or bacteria (E. coli or the like) transfected with ERRL1 cDNA and/or ERR cDNA. In addition, as the cells isolated from an animal
25 body, those of isolated from BAT, heart, skeletal muscle, kidney or the like, in which ERRL1 protein has been highly expressed, can be exemplified. On the other hand, in the case of using a non-human animal (such as mouse) as a target, a candidate substance is administrated to this animal body (systemically or locally) or is taken as
30 food or drink, then the foregoing requirements (a) to (d) are measured in

a tissue or cells of the animal. At this time, a transgenic non-human animal provided by this invention may be used as a positive control. In other words, because this transgenic non-human animal overexpresses ERRL1 protein and, as a result, highly expresses receptor ERR and MCAD genes, therefore, by measuring whether or not a wild-type mouse administered with a candidate substance fulfills the foregoing requirements as much as this transgenic animal does, an effect of the candidate substance can be judged more precisely. In addition, in the case of using a transgenic non-human animal or a wild-type animal as a target, a treatment of feeding with a high-fat diet, loading with physical exercises or the like, or amount of food intake, body weight, energy expenditure or the like can be included in the judging requirements.

The judgment of the foregoing requirements (a) to (d) can be carried out by measuring the expression of the respective genes by measuring the level of their transcriptional products (mRNA or protein) using a known method. For example, the judgment of the foregoing requirements (a) to (d) can be carried out by a known method such as in situ hybridization, Northern blotting, dot blotting, RNase protection assay, RT-PCR, Real-time PCR (Journal of Molecular Endocrinology, 25, 169-193 (2000) and the literatures cited therein), and DNA array analysis method (edited by Mark Shena, "Microarray Biochip Technology", Eaton Publishing (March 2000)). A screening of an active ingredient substance in a drug for obesity and/or diabetes using such a technique and a reagent, method, process, analysis program and the like to be used therein are all included in this invention. Specific examples of the judgment include, for example, the following methods.

(A) Screening of substance that increases expression level of ligand factor ERRL1

A reporter plasmid is constructed in which the promoter region of ERRL1 gene has been fused with cDNA of luciferase or β -galactosidase. After this reporter plasmid is transfected into cells, a candidate substance to be investigated is added to the cells and the activity of luciferase or β -galactosidase in the extracted solution of the cultured cells is measured after several days. If an increase of luciferase or β -galactosidase is observed by the addition of the candidate substance, the possibility is strongly suggested that this substance has an ability to activate the promoter of ERRL1 gene. Then, this candidate substance is added to cultured cells and an increase of mRNA expression level of ERRL1 gene is evaluated by the Northern blot method, quantitative RT-PCR method or the like. Similarly, using cultured cells, an increase of expression level of ERRL1 protein can be also evaluated by the Western blot method using an anti-ERRL1 antibody. Alternatively, a candidate substance is fed to a non-human animal and an increase of ERRL1 mRNA level or ERRL1 protein level in the cells isolated from the animal (e.g., BAT, heart, skeletal muscle, kidney or the like) may be evaluated in the same manner.

(B) Screening of substance that increases the transcriptional activity of nuclear receptor ERR

After the reporter plasmid of luciferase or β -galactosidase having an ERR responsive sequence or gal4 responsive sequence in the promoter region is cotransfected into cultured cells with an expression vector of the full-length ERR protein or a fused protein of the ligand binding region of ERR with the DNA binding region of gal4, respectively, a candidate substance to be investigated is added to the cells and the activity of luciferase or β -galactosidase in the extracted solution of the cultured cells is measured after several days. If an increase of luciferase or β -galactosidase is observed depending on the existence of

the responsive sequence by the addition of the candidate substance, the possibility is strongly suggested that this substance has an ability to activate the transcription of ERR gene. Then, this candidate substance is added to cultured cells and an increase of mRNA expression level of a target gene of ERR, for example MCAD gene, is evaluated by the Northern blot method, quantitative RT-PCR method or the like. Similarly, using cultured cells, an increase of expression level of the product of a target gene of ERR, for example MCAD protein, can be evaluated by the Western blot method using an anti-MCAD antibody. Alternatively, a candidate substance is fed to a non-human animal and an increase of MCAD mRNA level or MCAD protein level in the cells isolated from the animal (e.g., BAT, heart, skeletal muscle, kidney or the like) may be evaluated in the same manner.

(C) Screening of substance that promotes binding of ERRL1 to ERR

In yeast or cultured animal cells, using the two hybrid method, the binding of ERRL1 to ERR is quantified as the activity of luciferase or β -galactosidase. When a candidate substance to be investigated is added to the system, if luciferase or β -galactosidase is increased compared to the case where the substance has not been added, the possibility is strongly suggested that this substance has an ability to activate the binding of ERRL1 to ERR. Then, when this drug substance is added in an immunoprecipitation method or a GST-pull down assay, the level of coprecipitated ERRL1 and ERR proteins is evaluated by the Western blot method. On the other hand, in a gel retardation assay using ERR protein and an oligo DNA containing the ERR responsive sequence, according to the index that is based on whether or not the level of signal that super shifts in the presence of ERRL1 is increased by the addition of this candidate substance, it can be confirmed whether or not this substance has an effect on promoting the binding of ERRL1 to

ERR. Further, by adding this candidate substance to a detection assay system to detect interaction using an apparatus such as Biacore, it can be confirmed whether or not this substance has an effect on promoting the binding of ERRL1 to ERR.

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(D) Screening of substance that increases expression level of MCAD gene product

A reporter plasmid is constructed in which the promoter region of MCAD gene has been fused with cDNA of luciferase or β -galactosidase. After this reporter plasmid is transfected into cells, a candidate substance to be investigated is added to the cells and the activity of luciferase or β -galactosidase in the extracted solution of the cultured cells is measured after several days. If an increase of luciferase or β -galactosidase is observed by the addition of the candidate substance, the possibility is strongly suggested that this substance has an ability to activate the promoter of MCAD gene. Then, this candidate substance is added to cultured cells and an increase of mRNA expression level of MCAD gene is evaluated by the Northern blot method, quantitative RT-PCR method or the like. Similarly, using cultured cells, an increase of expression level of MCAD protein can be evaluated by the Western blot method using an anti-MCAD antibody. Alternatively, by the same method as in the foregoing (B), an increase of MCAD mRNA level or MCAD protein level may be evaluated in a non-human animal.

The genetic engineering techniques (recombinant DNA techniques) used in each of the foregoing methods can be carried out in accordance with the methods described in J. Sambrook, E. F. Fritsch & T. Maniatis, "Molecular Cloning: A Laboratory Manual (2nd edition)", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989); D. M. Glover et al. ed., "DNA Cloning", 2nd ed., Vol. 1 to 4, (The Practical

Approach Series), IRL Press, Oxford University Press (1995); Japanese Biochemical Society ed., "Zoku Seikagaku Jikken Kouza 1, Idenshi Kenkyuho II" Tokyo Kagaku Dozin (1986); Japanese Biochemical Society ed., "Shin Seikagaku Jikken Kouza 2, Kakusan III (Kumikae DNA Gijutsu)" Tokyo Kagaku Dozin (1992); R. Wu ed., "Methods in Enzymology", Vol. 68 (Recombinant DNA), Academic Press, New York (1980); R. Wu et al. ed., "Methods in Enzymology", Vol. 100 (Recombinant DNA, Part B) & 101 (Recombinant DNA, Part C), Academic Press, New York (1983); R. Wu et al. ed., "Methods in Enzymology", Vol. 153 (Recombinant DNA, Part D), 154 (Recombinant DNA, Part E) & 155 (Recombinant DNA, Part F), Academic Press, New York (1987), J. H. Miller ed., "Methods in Enzymology", Vol. 204, Academic Press, New York (1991); R. Wu et al., ed., "Methods in Enzymology", Vol. 218, Academic Press, New York (1993); S. Weissman ed., "Methods in Enzymology", Vol. 303, Academic Press, New York (1999); J. C. Glorioso et al., ed., "Methods in Enzymology", Vol. 306, Academic Press, New York (1999), etc. or the methods described in the references cited therein or substantially the same methods or the modifications thereof.

The anti-ERRL1 antibody to be used in the foregoing method (A) and the anti-MCAD antibody to be used in the foregoing methods (B) and (D) are a polyclonal antibody or a monoclonal antibody that recognizes either ERRL1 protein or MCAD protein, and include a whole molecule that can bind to the epitope of the respective proteins, and all the fragments of Fab, F(ab')₂, Fv and the like. Such an antibody can be obtained from serum after immunizing an animal by using a purified ERRL1 protein or MCAD protein, or a partial peptide thereof as an antigen. Alternatively, it can be prepared by introducing an expression vector for a eukaryotic cell into the muscle or the skin of an animal with an injector or a gene gun, and collecting the serum. As the animal,

mouse, rat, rabbit, goat, chicken or the like is used. If hybridoma is produced by fusing B cells collected from the spleen of an immunized animal with myeloma, a monoclonal antibody can be produced. Preparation of such an antibody can be carried out in accordance with a known method described in the literatures (Shin Seikagaku Jikken Kouza 12, Molecular Immunology III, Antigens, Antibodies, and Complements, Nancodo, 1992; "Monoclonal Antibody", co-authored by Komei Nagamune and Hiroshi Terada, Hirokawa Shoten, 1990; "Monoclonal Antibody", James W. Goding, third edition, Academic Press, 1996 and the like). Further, the measurement methods using such an antibody can be carried out by referring to the literatures, for example, Hiroshi Irie ed., "Radioimmunoassay" (published by Kodansha, 1974); Hiroshi Irie ed., "Radioimmunoassay; Second Series" (published by Kodansha, 1979); Eiji Ishikawa, et al. ed., "Enzyme Immunoassay" (published by Igaku Shoin, 1978); Eiji Ishikawa, et al. ed., "Enzyme Immunoassay" (Second Edition) (published by Igaku Shoin, 1982); Eiji Ishikawa, et al. ed., "Enzyme Immunoassay" (Third Edition) (published by Igaku Shoin, 1987); H. V. Vunakis et al. ed., "Methods in Enzymology", Vol. 70 (Immunochemical Techniques, Part A), Academic Press, New York (1980); J. J. Langone et al. ed., "Methods in Enzymology", Vol. 73 (Immunochemical Techniques, Part B), Academic Press, New York (1981); J. J. Langone et al. ed., "Methods in Enzymology", Vol. 74 (Immunochemical Techniques, Part C), Academic Press, New York (1981); J. J. Langone et al. ed., "Methods in Enzymology", Vol. 84 (Immunochemical Techniques, Part D: Selected Immunoassays), Academic Press, New York (1982); J. J. Langone et al. ed., "Methods in Enzymology", Vol. 92 (Immunochemical Techniques, Part E: Monoclonal Antibodies and General Immunoassay Methods), Academic Press, New York (1983); J. J. Langone et al. ed., "Methods in Enzymology", Vol. 121 (Immunochemical Techniques, Part I: Hybridoma Technology and

Monoclonal Antibodies), Academic Press, New York (1986); J. J. Langone et al. ed., "Methods in Enzymology", Vol. 178 (Antibodies, Antigens, and Molecular Mimicry), Academic Press, New York (1989); M. Wilchek et al. ed., "Methods in Enzymology", Vol. 184 (Avidin-Biotin Technology), Academic Press, New York (1990); J. J. Langone et al. ed., "Methods in Enzymology", Vol. 203 (Molecular Design and Modeling: Concepts and Applications, Part B: Antibodies and Antigens, Nucleic Acids, Polysaccharides, and Drugs), Academic Press, New York (1991) and the like, or the references cited therein.

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In the case where screening is carried out by using a non-human animal as a target in the foregoing methods (A), (B) and (D), a transgenic non-human animal provided by this invention can be used as a positive control. In other words, this transgenic non-human animal of this invention overexpresses ERRL1 protein and, as a result, it highly expresses receptor ERR and MCAD genes, thereby showing an anti-obesity and anti-diabetic property. Accordingly, the action of the candidate substance in the individual can be determined by using the gene expression in the cells of this transgenic animal as a control.

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The transgenic non-human animal in this invention can be produced in accordance with a known transgenic animal production method (e.g., Proc. Natl. Acad. Sci. USA 77; 7380-7384, 1980). In other words, the foregoing mouse ERRL1 polynucleotide or human ERRL1 (human PERC) polynucleotide (hereinafter, referred to as 'introduced gene' in some cases) is introduced into a totipotent cell of the non-human animal and this cell is developed until it becomes an individual. Then, by selecting an individual in which the introduced gene has been integrated into the genome of the somatic cell, the target transgenic non-human animal can be produced. As the non-human

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animal, animals used as a laboratory animal such as mouse, rat, rabbit, dog, cat and the like can be used as a target, however use of mouse is preferred because the inbred line has been established. In addition, a promoter sequence or an enhancer sequence is connected to the introduced gene to control its expression. Depending on the selection of this promoter/enhancer sequence, ERRL1 protein can be expressed systemically or it can be selectively expressed in a specific tissue. Such an introduced gene can be constructed by introducing and ligating the foregoing polynucleotide or promoter/ enhancer sequence to a circular DNA vector so as to be arranged in an effective position in the expression regulation of the introduced gene. Then, after this vector DNA is digested with a restriction enzyme, the one, from which the vector region has been removed, is introduced into a totipotent cell. As the totipotent cell to which a gene is introduced, a fertilized egg, an early embryo or an embryonic stem cell (ES cell) can be used. In addition, as the method of introducing a gene into a totipotent cell, when a ratio of generating transgenic animal individuals or a ratio of inheriting an introduced gene to the next generations are taken into account, a physical injection (microinjection) method of DNA is the most preferred. A fertilized egg, to which a gene is injected, is then transferred to the oviduct of a surrogate mother and after it is generated to an individual and born, the animal is grown with a foster parent and DNA is extracted from part of the body (e.g., tail tip), and the existence of the introduced gene is confirmed by the Southern analysis or the PCR method. Assuming that the individual in which the existence of the introduced gene has been confirmed is the first generation (founder), the introduced gene is inherited to 50% of its offspring (F1). Further, by crossing this F1 individual with a wild-type animal or other F1 animal, an individual (F2) having the introduced gene in one (heterozygote) or both (homozygote) of diploid chromosomes can be produced. The transgenic animal produced as above overexpresses

ERRL1 protein in all somatic cells or a specific tissue and, as described in the after-mentioned Examples, it has a unique characteristic of being lean although it is hyperphagic, and showing significantly high energy expenditure. Such a transgenic animal is useful not only as a control in the foregoing screening method but also as a model animal in the elucidation of an anti-obesity or anti-diabetic mechanism at the whole body level.

The invention of this application further provides a novel drug for obesity and/or diabetes comprising as the active ingredient at least one substance specified by the foregoing screening method. In other words, this drug has at least one of the actions selected from, firstly, the action of increasing the expression level of ERRL1, secondary, the action of increasing the transcriptional activity of ERR, thirdly, the action of increasing the interaction between ERRL1 and ERR, and fourthly, the action of increasing the expression level of an MCAD gene product.

The drug of this invention can be formulated by homogenously mixing the foregoing active ingredient and a pharmaceutically acceptable carrier. The carrier can be appropriately selected from a wide range according to the administration form of the drug. It is desired that the drug of this invention be in a unit dosage form that can be administered orally or by injection. The oral liquid preparation such as a suspension or syrup can be produced by using water, a sugar such as sucrose, sorbitol or fructose, a glycol such as polyethylene glycol, an oil such as sesame oil or soybean oil, an antiseptic such as alkyl parahydroxy benzoate, a flavor such as strawberry flavor or peppermint, or the like. A powder, pill, capsule and tablet can be formulated by using a diluent such as lactose, glucose, sucrose or mannitol, a disintegrating agent such as starch or sodium alginate, a lubricant such as magnesium

stearate or talc, a binder such as polyvinyl alcohol, hydroxypropyl cellulose and gelatin, a surface active agent such as a fatty acid ester, an elasticizer such as glycerin or the like. The tablet and capsule are a preferred unit dosage form in the formulation of this invention in that it is easy to administer. When the tablet or capsule is produced, a solid pharmaceutical carrier is used. In addition, the solution for injection can be formulated by using a carrier composed of a salt solution, glucose solution, or a mixture of a salt solution and glucose solution, a variety of buffer solutions or the like. Further, an injectable solution may be also prepared by formulating it in a powder state and then mixing it with the foregoing liquid carrier prior to its use.

Hereunder, the invention of this application will be explained in more detail and specifically by showing Examples, however, the invention of this application is not intended to be limited to the following examples.

Examples

1. Methods

1.1. Database search

An EST homology search was performed using the BLAST program (Altshul, S. F. et al., Nucleic Acids Res. 25: 3389-3402, 1997).

1.2. RNA analysis

Northern blot analyses were performed in accordance with the description in the literature (Sambrook and Maniatis, in Molecular Cloning-A Laboratory Manual, 7.2-7.87, Cold Spring Harbor Laboratory Press, New York, 1989). The cDNA probes for MCAD (Genbank

accession No. U07159), PRC (BC013720), ERR1 (U85259), ERR2 (S82458), ERR3 (AF117254), ACC2 (AF290178), and UCP-3 (AF032902) were obtained by RT-PCR.

5 1.3. Assays for transcriptional activity

Transfections and reporter assays were performed in accordance with the description in the literature (Takada, I. et al., *Mol. Endocrinol*, 14: 733-740, 2000). More specifically, a reporter gene containing four copies of a GAL4 binding sequence ((UAS)₄-Luc) was transfected into
10 CV-1 cells in the presence or absence of a chimeric receptor expression vector in which the DNA binding region of GAL4 had been fused with the ligand binding region for the nuclear receptor (pCMX-GAL4-nuclear receptor), and the transcriptional activity of GAL4-nuclear receptor-fused protein was measured (Takada, I. et al., *Mol. Endocrinol*, 14: 733-740,
15 2000). All luciferase activities were normalized by the co-transfected β -galactosidase activities. Amino acids 1-147 of GAL4 fused to the ligand binding domain for the following nuclear receptors were used: mouse AR (amino acids, 607-899, Genbank accession number, X59592), human ER α (a.a. 251-595, X03635), human GR (a.a. 489-777, M10901),
20 rat FXR (a.a. 190-469, U18374), human RAR α (a.a. 126-432, X06538), human RXR α (a.a. 222-462, X52773), mouse PPAR α (a.a. 156-468, X57638), human PPAR γ 1 (a.a. 176-478, L40904), human PXR (a.a. 110-434, AF084645), human ERR1 (a.a. 147-422, L38487), human ERR2 (a.a. 171-433, X51417), human ERR3 (a.a. 173-436, AF058291), human
25 HNF4 α (a.a. 125-465, X76930), human NOR (a.a. 361-626, D78579), human NURR1 (a.a. 264-535, S77154), human ROR α 1 (a.a. 140-523, U04897), human SF1 (a.a. 64-461, U76388), human COUP (a.a. 156-423, X12795), human TR2-11 (a.a. 149-603, M29960), human RevErbA (a.a. 199-614, M24898). The abbreviations of nuclear receptors are described
30 in the references of respective Genbank files (Giguere, V., *Endocr. Rev.*

20: 689-725, 1999). GAL4-nuclear receptor expression plasmids were provided from Dr. K. Umesono. Mouse PGC-1 cDNA was obtained by screening a mouse embryo cDNA library. The full length cDNA of human ERR1, 2 and 3 were obtained by RT/PCR. The amplified products were subcloned into pCAGGS and pCMX expression vectors, and confirmed by sequencing.

1.4. Protein interaction assays

In accordance with the description in the literature (Kamei, Y. et al., Cell 85: 403-414, 1996), the constructs (expression vectors) of GST fused with ERR1 and ERR3 were incubated with ³⁵S-ERRL1 (TNT, Promega) and washed. Then, the bound ³⁵S-ERRL1 was separated by SDS-PAGE, and quantified by autoradiography.

1.5. Stable cell lines

Phoenix 293 cells (gifted from Dr. G. P. Nolan, University of Stanford) were used for the retrovirus packaging (Grignani, F. et al., Cancer Res. 58: 14-19, 1998). pLNCX-derived expression plasmid (Clontech) containing cDNA of ERR1, ERR3 or GFP (control) and pMX-derived expression plasmid (Misawa, K. et al., Proc. Natl. Acad. Sci. USA 97: 3062-3066, 2000) containing cDNA of ERRL1, PGC-1 or GFP were used in accordance with the manufacturer's instruction.

1.6. Generation of transgenic mice

ERRL1 cDNA was cloned into pCAGGS (Niwa, H., Gene 108: 193-200, 1991), the transgene (Fig. 4A) was excised and purified (2 ng μ l⁻¹). Fertilized eggs were recovered from BDF1 females (C57BL/6xDBA/2) crossed with BDF1 males and microinjected with the transgene by the standard method (Gordon, J., in Guide to Techniques in Mouse Development, (eds Wassarman, P. M. & DePamplilis, M. L.,

747-771, Academic press, San Diego, 1993). Care of the mice was performed in accordance with the guideline of the institution to which the inventors belong.

5 1.7. High-fat diet

Mice were fed with a regular chow diet (Oriental Yeast Inc., Tokyo, Japan) or a high-fat diet (Aoki, N. et al., Obesity Res. 1: 126-131, 1993) which contained casein (20% wt/wt), α -cornstarch (30.2%), sucrose (10%), lard (25%), corn oil (5%), minerals (3.5%), vitamins (1%), cellulose
10 powder (5%), and D,L-methionine (0.3%).

1.8. Determination of energy expenditure

Oxygen consumption and carbon dioxide production were determined by using an indirect calorimeter system composed of a mass
15 analyzer and a computer (Komenani, N. et al., J. Nutr. Sci. Vitaminol. (Tokyo) 41: 395-407, 1995). Mice were individually placed in an open-circuit plastic respiration chamber (24 x 46 x 18 cm) connected to a gas mass spectrometer (WSMR-1400, Westron, Chiba, Japan). The airflow was controlled at 2 l/min. Gas analysis was carried out from
20 10:00 to 9:00 on the following day. Samples were monitored continuously in 2 minute blocks in room air as a reference. Locomotor activity was recorded automatically every 10 minutes by an Animex-III (Shimadzu, Kyoto, Japan) laid under each respiration chamber. Energy expenditure was calculated in accordance with the description in the
25 literature (Komenani, N. et al., J. Nutr. Sci. Vitaminol. (Tokyo) 41: 395-407, 1995).

1.9. KKAY mice

KKAY mice were purchased from Clea Japan Inc. (Tokyo).

1.10. Statistical analyses

Statistical comparisons of data from two experimental groups were made by using Student's t-test. Comparison of data from multiple groups was made by one-way analysis of variance (ANOVA), and each group was compared with the other groups by the Fisher's protected least significant difference (PLSD) test (Statview 4.0, Abacus Concepts). Statistical significance was defined as $P < 0.05$ or $P < 0.01$.

2. Results

2.1. Cloning of ERRL1 cDNA and its features

As a result of searching EST (expression sequence tags) for a PGC-1-related molecule, EST having an extremely high homology to PGC-1 was found, and the full length cDNA containing this EST was isolated. This cDNA consists of about 3.4 kb (SEQ ID NO: 1), and encodes a protein consisting of 1,014 amino acid sequences (sequence number 2). This protein was named ERRL1 (abbreviation of ERR ligand 1) based on its properties as a 'protein ligand' for ERRs (as shown later in detail). Incidentally, Lin et al. reported the cloning of a PGC-1 homologue named PGC-1 β (Lin, J. et al., J. Biol. Chem. 277: 1645-1648, 2002), however, PGC-1 β only has one amino acid difference from ERRL1 (The 260th leucine in the sequence number 2 is a proline in the PGC-1 β). ERRL1 and PGC-1 show a high degree of amino acid identity except for the central unique region in ERRL1. The homologous region can be divided into five domains based on sequence identity and predicted functional properties (Fig. 1). The N-terminal region of ERRL1 (amino acids 1-282) contains two LXXLL motifs, which are a proposed binding motif to nuclear receptors (Torchia, J. et al., Nature, 387: 677-684, 1997; Heery, D. M., et al., Nature, 387: 733-736, 1997), and has 41%

identity with PGC-1. The second region (amino acids 283-579), which is unique to ERRL1, contains E (glutamic acid) repeats and one LXXLL motif. The third region (amino acids 580-656) is highly conserved (47% amino acid identity). The fourth region (amino acids 657-882) is less conserved (22% amino acid identity) and contains a very short serine/arginine (SR)-rich domain (Tacke, R. & Manley, J. L. Curr. Opin. Cell Biol. 11: 358-362, 1999) in ERRL1 compared with the long SR domain in PGC-1. The C-terminal domain (amino acids 883-1014) has a putative RNA-binding domain (Krecic, A. M. & Swanson, M. S., Curr. Opin. Cell Biol. 11: 363-371, 1999) and is highly conserved (52% amino acid identity).

2.2. Similar expression patterns between ERRL1 and ERR

The expression patterns of ERRL1 in different tissues from adult mice were examined. Two ERRL1 mRNAs of about 10 kb and 4 kb in length were observed. ERRL1 mRNAs were abundant in brain, BAT, heart and skeletal muscle, and were also detected in kidney, stomach and white adipose tissue (WAT). Consistent with a previous report (Puigserver, P. et al., Cell 92: 829-839, 1998), PGC-1 expression was increased in mouse BAT after exposure to cold stress, however, the expression of ERRL1 mRNAs was only marginally up-regulated by this stress.

Further, the expression pattern of ERRL1 closely resembles that of ERR1 (Sladek, R. et al., Mol. Cell. Biol. 17: 5400-5409, 1997 and Fig. 2), with both mRNAs being highly expressed in tissues that can use lipids as a source of cellular energy, e.g., BAT, heart, skeletal muscle, and kidney (Fig. 2A). ERR2 mRNA expression was not be detected, which is consistent with a previous study report (Giguere, V. et al., Nature 331: 91-94, 1988).

Then, it was examined whether ERRL1 expression is augmented

during adipocyte differentiation of 3T3-L1 cells. It is certain that ERRL1 mRNAs were present at very low levels in 3T3-L1 preadipocytes and were markedly induced during adipocyte differentiation. In contrast, mRNAs of PGC-1 and PRC, another PGC-1-related molecule (Andersson, U. & Scarpulla, R. C. Mol. Cell. Biol. 21: 3738-3749, 2001) remained at low levels during this adipocyte differentiation (Fig. 2B). Similar augmentation of ERRL1 mRNAs was observed in mature adipocytes (Smas, C. M. & Sul, H. S. Biochem. J. 309: 697-710, 1995) differentiated from another preadipocytes, 10T1/2 cells (Fig. 2C). Further, expression profiles of ERR1 and its target MCAD were quite similar to that of ERRL1 (Figs. 2B and 2C), however, ERR2 mRNA and ERR3 mRNA expressions were not detected in these cells. These results suggest that ERRL1 is involved in the gene regulations in mature adipocytes-related functions such as the lipid metabolism.

2.3. PGC-1 as a general nuclear receptor agonist.

It was examined that whether ERRL1 can function as a coactivator of PPAR γ , using PGC-1 as a control coactivator. Contrary to our expectations, any evidence indicating that ERRL1 could activate PPAR γ -mediated transcription could not be found out. In the control experiments, it was confirmed that the expression of PGC-1 on its own was able to drastically activate PPAR γ -mediated transcription without the addition of any exogenous lipophilic ligands. PGC-1 is known to physically interact not only with PPAR γ but also with several other nuclear receptors (Puigserver, P. et al., Cell 92: 829-839, 1998; Yoon, J. C. et al., Nature 413: 131-138, 2001; Tcherepanova, I. et al., J. Biol. Chem. 275: 16302-16308, 2000; Vega, R. B. et al., Mol. Cell. Biol. 20: 1868-1876, 2000; Delerive, P. et al., J. Biol. Chem. 277: 3913-3917, 2002). Furthermore, PGC-1 has been shown to be induced by certain environmental changes such as cold exposure (Puigserver, P. et al., Cell

92: 829-839, 1998) and fasting (Yoon, J. C. et al., Nature 413: 131-138, 2001; Lehman, J. J. et al., J. Clin. Invest. 106: 847-856, 2000). These observations raise the possibility that the expression of PGC-1 alone can activate multiple nuclear receptor pathways. This putative character is analogous to classical lipophilic ligands for the nuclear receptors. To address this possibility, nuclear receptors whose DNA-binding domains were replaced by the GAL4 DNA-binding domain were used. By this replacement, the comparison of transcriptional activity profiles of multiple nuclear receptors with the same reporter (gene) such as (UAS)₄-Luc can be easily performed. Indeed, PGC-1 was able to activate transcription by means of multiple nuclear receptors including orphan HNF4 α , SF1, and ERRs. Among these, PGC-1 most strongly activated HNF4 α , which is consistent with a recent report (Yoon, J. C. et al., Nature 413: 131-138, 2001). Subsequently, PGC-1 activated ER α , SF1, ERRs, PPARs, PXR, RAR α , and RXR α (Fig. 3A). From these observations, it was confirmed that PGC-1 can function as a general and broad nuclear receptor agonist.

2.4. ERRL1 as an ERR protein ligand

Using the same GAL4-fused nuclear receptor set as above, a potential partner of ERRL1 was searched, and it was found out that ERRL1 specifically activated ERR-mediated transcription. ERR3 was most strongly activated by ERRL1, followed by ERR1 and ERR2 (Fig. 3B). Then, the transcriptional activation properties of ERRL1 on full-length ERRs were tested. Consistent with the experiments above, ERRL1 dose-dependently activated the full-length ERR3- and the full-length ERR1-mediated transcription by means of the ERR responsive sequence (ERRE) of the MCAD gene promoter (Sladeck, R. et al., Mol. Cell. Biol. 17: 5400-5409, 1997; Vega, R. B. & Kelly, D. P. J. Biol. Chem. 272: 31693-31699, 1997) (Fig. 3C). The former was activated much more

strongly than the latter.

Next, it was tested whether ERRL1 physically interacts with ERRs by in vitro binding analysis with glutathione S-transferase-fused ERR (GST-ERR) produced by using bacteria and ERRL1 translated in vitro. Matrix-bound GST-ERR1 and GST-ERR3, but not GST alone, retained radiolabeled ERRL1 efficiently (Fig. 3D). As reported previously (Sladek, R. et al., *Mol. Cell. Biol.* 17: 5400-5409, 1997; Vega, R. B. & Kelly, D. P. *J. Biol. Chem.* 272: 31693-31699, 1997), it was confirmed that ERRs had been bound to radiolabeled MCAD ERRE oligonucleotides in a gel mobility shift assay, and that the ERR-DNA complexes were supershifted by the addition of the ERRL1 protein. Given that lipophilic hormones were not added either in the synthesis of the proteins or in the binding reactions, these results indicate that ERRL1 and ERRs can directly interact with the ERR target promoter DNAs.

Next, ERRL1 and ERR were expressed in 10T1/2 cell line, and it was examined whether ERRL1 can activate MCAD gene expression via the ERR. 10T1/2 cells were infected with several combinations of recombinant retroviruses, each containing a DNA encoding GFP (control), ERR1, ERR3, ERRL1 or PGC-1. Northern blot analysis was performed to confirm the expressions of the transduced genes in each cell line (Fig. 3E). The sole expression of either ERR1 or ERR3 led to a modest increase of MCAD mRNA level compared with that in cells infected with GFP alone. Coexpression of both ERR1 and ERRL1 or ERR3 and ERRL1 led to a further significant increase of the MCAD mRNA level (Fig. 3E). These results collectively demonstrate that ERRL1 can function as a 'protein ligand' for ERRs and activate ERR-mediated transcription at least in cultured cells.

2.5. Creation of ERRL1 mice

In order to examine the effects of increased ERRL1 expression in

vivo, ERRL1 transgenic mice were created. It was expected that these mice would exhibit phenotypes that mimic those induced by activated ERR-mediated transcription. The chicken β -actin promoter with the cytomegalovirus immediate early enhancer (CAG promoter) was used to promote the expression of the mouse ERRL1 transgene in mice (Fig. 4A). Southern blot analysis of tail DNAs of the mice was performed to determine the transgene copy numbers (Fig. 4B).

Expression of the ERRL1 transgene was evaluated by Northern blot analysis on RNAs isolated from tissues of ERRL1 mice and control littermate mouse at 8 weeks of age (Fig. 4C). When a transgene-specific probe (Probe 2, Fig. 4A) was used, only a single 4-kb band was detected. The CAG promoter has been reported to show a strong activity in any tissues (Niwa, H. et al., *Gene* 108: 193-200, 1991). Unexpectedly, however, the use of this promoter resulted in high expression levels of the ERRL1 transgene in several restricted tissues such as brain, BAT, heart, skeletal muscle, and testis, but a very low-level of expression in liver (Fig. 4C). These expression profiles corresponded relatively well to the endogenous ERRL1 expression patterns (Fig. 4C). These observations suggest that tissue-specific regulatory cis-sequences crucial for ERRL1 expression may reside in the ERRL1 cDNA used in this construct.

As predicted from the in vitro data, the increased expression of ERRL1 in the skeletal muscle of ERRL1 mice (Fig. 4D) enhanced MCAD mRNA expression in vivo. In contrast, the increased expression of ERRL1 did not affect the gene expression of acetyl-CoA carboxylase 2 (ACC2) (Abu-Elheiga, L. et al., *Science* 291: 2613-2616, 2001) or uncoupling protein-3 (UCP3) (Clapham, J. C. et al., *Nature* 406: 415-418, 2000).

2.6. ERRL1 mice are hyperphagic but lean

The apparent phenotype observed in ERRL1 mice was lean, which agreed well with those predicted from increased β -oxidation of fatty acids. This phenotype was more prominent when mice were fed with a high-fat diet (Aoki, N. et al., Obesity Res. 1: 126-131, 1993). Groups of six male wild-type control mice and six male ERRL1 mice, at 9 weeks of age, were freely fed with a high-fat diet, and the food consumption and body weight of each mouse were measured every week. ERRL1 mice consumed significantly more food than the control mice (Fig. 5A). However, the transgenic mice weighed 15 to 25% less than the control mice before and throughout the feeding periods with a high-fat diet (Fig. 5B), and they accumulated significantly less fat in their adipose tissues (Fig. 5C). The epididymal WAT in the ERRL1 mice only weighed 0.92 ± 0.28 g, compared with 2.0 ± 0.30 g in the control mice (Fig.). In contrast, liver weights were not significantly different (ERRL1 mice: 1.23 ± 0.06 g; control mice: 1.4 ± 0.08 g). The adipocytes of the transgenic mice were smaller than those of the control mice (average diameter of adipocytes in ERRL1 mice: 25.1 ± 1.1 μ m; control mice: 54.5 ± 2.2 μ m; Figs. 5E and 5F).

The blood was collected from these mice, and serum components were biochemically analyzed. The decrease in adipose mass resulted in a decrease in the amount of leptin in the serum (ERRL1 mice: 5.9 ± 3.5 ng ml⁻¹, control mice: 25.0 ± 6.6 ng ml⁻¹). As leptin is an anti-appetite hormone secreted from WAT (Friedman, J. M. Nature 404: 632-634, 2000), the decreased level of leptin observed in ERRL1 mice is consistent with the observation that they are hyperphagic. Furthermore, decreased level of insulin were observed in the transgenic mice compared with the control mice fed with a high-fat diet. Mice, when fed with a high-fat diet, generally develop resistance to glucose uptake, even with a high plasma insulin concentration (Li, B. et al., Nat. Med. 6: 1115-1120, 2000). A similar state, which is called insulin resistance, is well observed in

humans with obesity or who are suffering from type 2 diabetes (Lovejoy, J. C. Curr. Atheroscler. Rep. 1: 215-220, 1999). The decreased body weight and decreased insulin level observed in ERRL1 mice indicate that the increased expression of ERRL1 can antagonize obesity and contribute to an improvement of diabetic state, even taking a high-calorie diet through overcoming insulin resistance.

2.7. Increased energy expenditure of ERRL1 mice

The energy expenditure of ERRL1 mice was examined. Gas analyses were carried out by using test chambers for respiration analysis, each containing a single mouse, and energy expenditure was calculated. As a result, the energy expenditures of ERRL1 mice were significantly higher than those of control mice at 12 weeks of age (resting energy expenditure: ERRL1 mice, 126.3 ± 3.8 kcal day⁻¹ kg^{-0.75}; control mice, 101.5 ± 6.7 kcal day⁻¹ kg^{-0.75}; $P < 0.05$, $n = 6$, per group; and total energy expenditure: ERRL1 mice, 211.2 ± 10.7 kcal day⁻¹ kg^{-0.75}; control mice, 155.9 ± 7.8 kcal day⁻¹ kg^{-0.75}; $P < 0.01$, $n = 6$, per group) (Figs. 5G and 5H). However, locomotor activity was not significantly different (ERRL1 mice, $7,338 \pm 700$ counts; control mice, $5,338 \pm 390$ counts, the data represent the sum of all transits over a 24 h period). These results indicate that the differences in body weights between the control mice and the ERRL1 mice are most likely to be due to a difference in their energy expenditure.

2.8. ERRL1 expression can antagonize a genetically programmed obesity

ERRL1 mice and KKAY mice were bred, and it was examined whether the obese phenotype of KKAY mice was counteracted by the increased expression of ERRL1. KKAY mice have a mutation of Ay, which leads to ectopic expression of the agouti coat-color protein, causing a dominantly inherited syndrome of obesity and yellow fur

(Siracusa, L. D., Trends Genet. 10: 423-428, 1994). The agouti protein acts as an antagonist for the melanocortine receptors, which are considered to act as a repressor receptor of appetite in the central nervous system (Friedman, J. M. Nature 404: 632-634, 2000; Siracusa, L. D., Trends Genet. 10: 423-428, 1994). KKAY males were bred with ERRL1 females, and their offsprings were selected by hair color (yellow, KKAY(+); black, KKAY(-)) and ERRL1 transgene expression. Comparison of the body weights of each group revealed that the ERRL1 transgene significantly suppressed the body weight increases of KKAY(+) mice (Figs. 6A and 6B), demonstrating that increased ERRL1 expression can antagonize obesity caused by a genetic abnormality.

3. Discussion

From the above results, it was confirmed that ERRL1 functions as a 'protein ligand' for ERRs and controls energy expenditure in vivo. In other words, this indicates that by increasing the expression level of ERRL1 in, for example skeletal muscle, this functions as a ERR protein ligand this time, and activates MCAD gene expression to lead to activation of fatty acid β -oxidation. When fatty acid β -oxidation is increased, as a result, accumulated fat will be decreased. It is predicted that the same effects can be achieved by activating the transcriptional ability of ERR and by increasing MCAD gene expression. These effects are very similar to a physiological state which is achieved by physical exercises.

In addition, the above results clearly indicate that the body weight can be decreased by pharmacological activation of ERRL1 or ERR while maintaining the usual calorie intake or more.

Industrial Applicability

As described in detail above, according to the invention of this application, there are provided a method of screening an ingredient in a remedy for obesity or diabetes by using the interaction between a nuclear orphan receptor ERR and its ligand molecule ERRL1 as an index and a novel drug containing a substance specified by this method as the active ingredient.

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